BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

{Exhibit 6}

Gall and Pardue, "Formation and Detection of RNA-DNA Hybrid Molecules in Cytological Preparations," Genetics 63: 378-383

Volume 63 June 1969 Number 2

Proceedings of the National Academy of Sciences

of the United States of America

*FORMATION AND DETECTION OF RNA-DNA HYBRID MOLECULES IN CYTOLOGICAL PREPARATIONS**

By Joseph G. Gall and Mary Lou Pardue

KLINE BIOLOGY TOWER, YALE UNIVERSITY

Communicated by Norman H. Giles, March 27, 1969

Abstract.—A technique is described for forming molecular hybrids between RNA in solution and the DNA of intact cytological preparations. Cells in a conventional tissue squash are immobilized under a thin layer of agar. Next they are treated with alkali to denature the DNA and then incubated with tritium-labeled RNA. The hybrids are detected by autoradiography. The technique is illustrated by the hybridization of ribosomal RNA to the amplified ribosomal genes in oocytes of the toad *Xenopus*. A low level of gene amplification was also detected in premeiotic nuclei (oogonia).

Several techniques are currently used for annealing RNA molecules to their complementary DNA sequences. For certain purposes both the RNA and DNA can be in solution,[1, 2] but it is often more convenient to have the DNA immobilized in a solid or semisolid matrix,[3, 4] or attached to a nitrocellulose membrane filter.[5] The hybrids are generally detected by scintillation counting of radioactive RNA after treatment with ribonuclease to remove unhybridized RNA.

The hybridization of RNA to the DNA in a cytological preparation should exhibit a high degree of spatial localization, since each RNA species hybridizes only with sequences to which it is complementary. The general principles of a cytological hybridization technique are not difficult to lay down. The chromosomes or nucleus should be fixed in as lifelike a fashion as possible; basic proteins should be removed, since they are known to interfere with the hybridization procedure;[5] the DNA should be denatured in such a way that cytological integrity is not lost; the hybridization should be carried out with radioactive RNA of very high specific activity, since the number of hybridized molecules at a given locus will be small; and detection should be by tritium autoradiography to permit maximal cytological resolution.

This communication describes a cytological hybridization technique applicable to conventional squash preparations. It is illustrated by the hybridization of rRNA to the extrachromosomal rDNA in oocytes of the toad *Xenopus*. A preliminary report on the technique was presented in December 1968 at the International Symposium of Nuclear Physiology and Differentiation, Belo Horizonte, Brazil.[6]

Materials and Methods.—The cytological hybridization technique combines certain features of the agar column[4] and filter methods.[5] It should be generally applicable to any material that can be examined as a squash or smear. The following procedure was used in making the preparation shown in Figure 1.

(1) Ovaries from recently metamorphosed *Xenopus laevis* were fixed for a few minutes in ethanol-acetic acid (3:1).

(2) The tissue was transferred to a drop of 45% acetic acid on a microscope slide and

teased with jewelers' forceps. The larger bits of tissue were removed, a cover slip was added, and the cells were squashed. The slides had been previously subbed by dipping into a solution of 0.1% gelatin in 0.1% chrome alum and draining until dry.

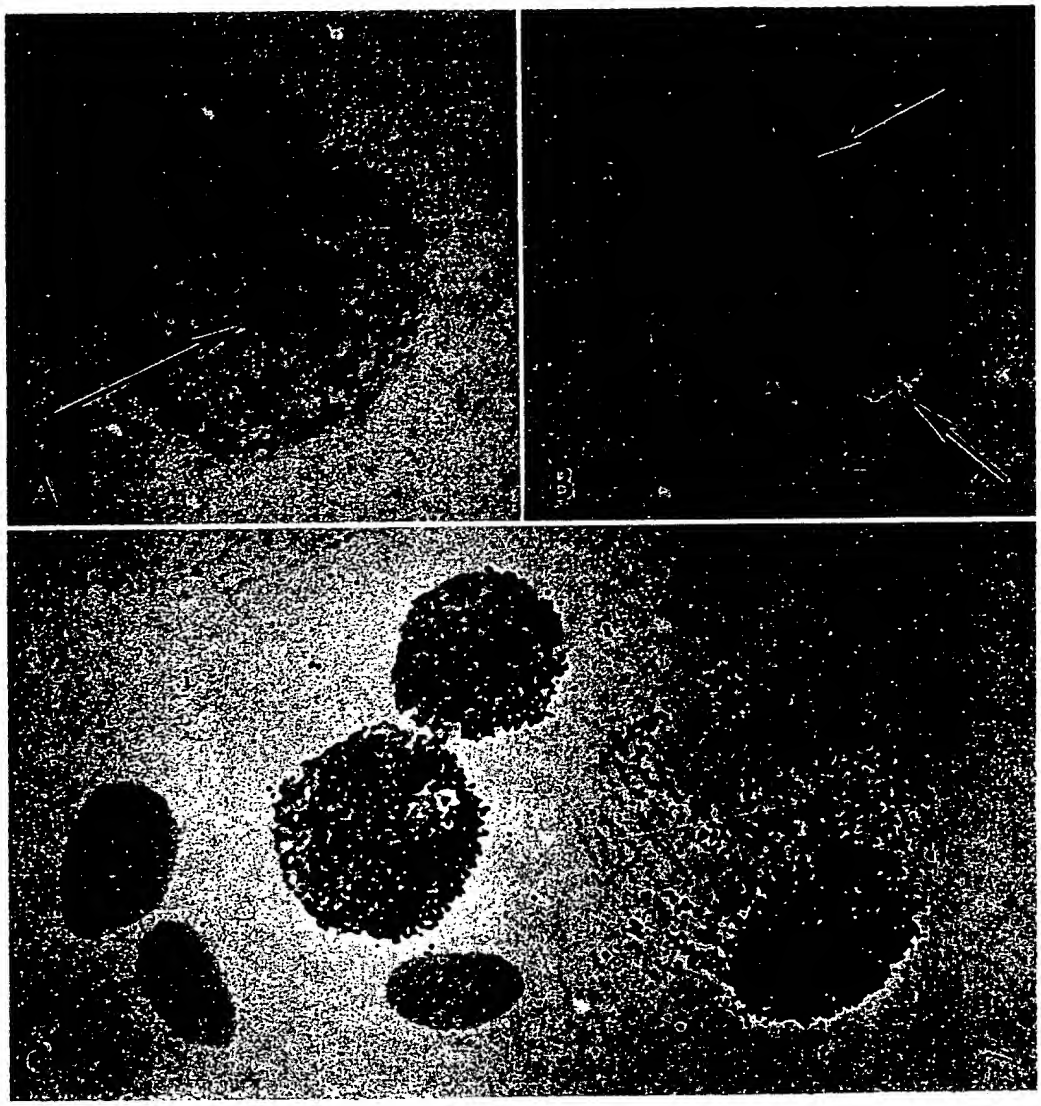

FIG. 1.—Autoradiographs of nuclei from the ovary of the toad *Xenopus*, after *in situ* hybridization with radioactive ribosomal RNA. The preparation was covered with agar, denatured in 0.07 *N* NaOH for 2 min, and hybridized with rRNA having a specific activity of 200,000 cpm/μg. Details are given in *Materials and Methods*. All nuclei from the same slide, exposed for 52 days, stained with Giemsa.

(*A*) Oogonial nucleus showing silver grains above the centrally placed nucleolus (*arrow*). This number of grains indicates that the nucleus contains some 20–40 copies of the nucleolus organizer. ×1500.

(*B*) Two leptotene nuclei with silver grains located over the eccentrically placed nucleolus (*arrows*). ×1500.

(*C*) Three unlabeled follicle nuclei and three labeled pachytene nuclei. The pachytene nuclei illustrate the progressive increase in extrachromosomal rDNA that occurs as the nucleus enlarges. The largest pachytene nucleus contains 25–30 pg of rDNA (approximately 3000 nucleolus organizers). The technique is not sensitive enough to demonstrate the small amount of rDNA in the two nucleolus organizers of the diploid follicle nuclei. ×1200.

(3) The slide was frozen on dry ice, and the cover slip was removed with a razor blade.[7] The slides were transferred to 95% ethanol for a few moments and then dried in air.

(4) The slides were dipped in 0.5% agar held molten at 60°C in a water bath. They were removed and drained vertically at room temperature. In this way a very thin but uniform agar layer covered the slide. The agar was allowed to gel but not to dry completely before the next step.

(5) The slide was placed for 2 min in 0.07 *N* NaOH at room temperature to denature the DNA. It was then transferred to 70 and 95% ethanol for a few minutes each and dried in air. Slides were often stored at this point.

(6) For the hybridization step about 200 μl of rRNA solution was placed directly onto the slide, and a large cover slip was added. The preparation was incubated at 66°C for 12 hr or longer in a moist chamber made from a Petri plate. We used 4-inch plastic plates into which were placed a few sheets of filter paper and enough 6X SSC to moisten thoroughly. The slide was supported above this on two rubber grommets. We used mixed 28*S* and 18*S* rRNA at a concentration of 1–2 μg/ml in 6X SSC. The rRNA was extracted by a detergent-phenol procedure from cultures of *Xenopus* cells[8] and was purified by sucrose density gradient centrifugation. It had a specific activity of 2×10^5 cpm/μg. The specific activity was determined by spotting known amounts of RNA-H^3 on nitrocellulose filters, drying, and counting in toluene-PPO-POPOP in a scintillation counter. The counter had an efficiency of about 40% for *unquenched* H^3 samples.

(7) After incubation the slides were washed in 6X SSC and placed in RNase for 1 hr at room temperature. Pancreatic RNase (1 mg/ml in 0.02 *M* Na acetate, pH 5) was boiled 5 min to remove protease activity and then made up to 20 μg/ml in 2X SSC.

(8) The slides were rinsed in 6X SSC and then in 70 and 95% ethanol before air-drying.

(9) The preparations were covered with Kodak NTB-2 liquid emulsion diluted 1:1 with distilled water. They were developed for 2 min in Kodak D-19, rinsed briefly in 2% acetic acid, and fixed 2 min in Kodak Fixer. After they were rinsed for 10 min in several changes of distilled water, they were stained 10 min with Giemsa, rinsed in distilled water, and air-dried. A drop of Permount medium and a cover slip were added.

Results.—The development of the cytological hybridization technique was facilitated by the use of oocytes of the toad *Xenopus laevis* as a test object. During pachytene of meiosis these cells carry out a differential synthesis of the genes coding for ribosomal RNA.[6, 9–12] Each pachytene nucleus, which contains 12 picograms (pg) of chromosomal DNA, produces about 30 pg of extrachromosomal rDNA. The extra DNA is cytologically detectable as a densely staining cap on one side of the nucleus. During diplotene the rDNA spreads over the inner surface of the nuclear envelope, where it produces the multiple nucleoli that characterize these cells.

The ovaries of recently metamorphosed toads contain many oocytes in the early meiotic stages. Squashes of these ovaries were denatured, hybridized with tritium-labeled rRNA, and subsequently autoradiographed. Heavy label was found in the oocyte nuclei, where it was limited to the extra DNA. Figure 1 (*B* and *C*) shows several stages in the formation of the nuclear cap from leptotene to late pachytene. In each case the label follows the distribution of the extra DNA, the chromosomes being without detectable radioactivity. The nuclei of follicle cells, connective tissue, and red blood cells are unlabeled (Fig. 1*C*), presumably because the technique is not sufficiently sensitive to demonstrate the small amount of rDNA in the normal genome. The unlabeled DNA in these nuclei provides a useful built-in control of the specificity of the hybridization reaction.

Attempts were made to hybridize preparations that had not been denatured

with alkali. In autoradiographs exposed for one or two weeks, such control preparations showed no detectable radioactivity in any nuclei. However, most control slides showed weak labeling when exposed for periods of one to two months. In these cases the label displayed the same specific localization seen in alkali-denatured slides, namely, over the rDNA of oocytes. These results suggest that a small amount of DNA is denatured during the fixing and squashing steps.

Two additional tests of specificity have been made. Ovary squashes were treated with DNase (0.3 mg/ml in 0.01 *M* Tris buffer containing 10^{-3} *M* $MgCl_2$, pH 7.2, 37°C, 3 hr). Some were stained with the Feulgen reaction to assess the removal of DNA, and the remainder were covered with agar and hybridized as usual. The preparations showed no detectable Feulgen stain and they gave negative autoradiographs, an indication of the failure to bind rRNA. Some protein remains in such preparations, since cytological details are visible either by phase contrast microscopy or after staining with fast green at pH 2.

We have found that a large excess of heterologous rRNA has no effect on the hybridization reaction (Table 1). Hybridizations were carried out with 2 μg/ml of radioactive *Xenopus* rRNA in the presence of nonradioactive rRNA from *Xenopus* or *Escherichia coli*. The *E. coli* rRNA had no effect on the binding of radioactive *Xenopus* rRNA, even when present at 800 μg/ml. By contrast, nonradioactive *Xenopus* rRNA reduced the binding to low levels. A small fraction of the radioactivity (8%) was not competed by homologous *Xenopus* rRNA. We do not know why competition was incomplete, although it should be noted that the radioactive rRNA was derived from cultured kidney cells, whereas the nonradioactive rRNA came from mature ovaries.

In order to assess the sensitivity of the hybridization procedure, we exposed preparations for periods up to two months. Our aim was to detect the earliest stages of rDNA amplification, which was thought to begin in leptotene or early pachytene.[11, 12] In such preparations the mid- and late pachytene nuclei showed total film blackening above the extra DNA. We were surprised to find easily detectable label not only over all leptotene nuclei (Fig. 1*B*), but also over most oogonial nuclei (Fig. 1*A*). In the leptotene nuclei the label was generally

TABLE 1. *Hybridization of Xenopus rRNA-H^3 in the presence of excess nonradioactive rRNA.*

Competing unlabeled rRNA	Silver grains per nucleus after 28 hr exposure, background subtracted (mean ± SEM)	Nuclei counted (no.)
None	185 ± 20	19 (4 preparations)
50–800 μg/ml *E. coli*	221 ± 10	43 (9 preparations)
50–800 μg/ml *Xenopus*	15 ± 2	50 (10 preparations)

In each case 2 μg/ml of *Xenopus* rRNA-H^3 was present during the hybridization step. *E. coli* rRNA showed no competition even at 800 μg/ml. Unlabeled *Xenopus* rRNA reduced the binding of radioactive rRNA to about 8% of the control value. The small residual binding was unrelated to the quantity of competing rRNA at the levels used here (50–800 μg/ml). The labeled *Xenopus* rRNA was from cultured kidney cells, whereas the unlabeled rRNA came from mature ovaries.

localized at the periphery of the nucleus, whereas in the oogonia it was more often found in one or two clusters within the nucleus. The distribution of silver grains parallels the position of the nucleoli in both cases. These results indicate that a low level of rDNA amplification is present in premeiotic nuclei.

We have begun to examine variables affecting the level of hybridization. The NaOH concentration in the denaturing step has been varied from 0.01 to 0.10 *N*. Concentrations of 0.01–0.02 *N* have given either no hybridization or only a low level. We presume that this is due to inadequate denaturing during the two-minute treatment. Concentrations of 0.05, 0.07, and 0.10 *N* gave roughly comparable levels of hybridization. However, the morphological disruption in preparations treated with 0.10 *N* NaOH was often extensive.

We have had some success in replacing the agar with a collodion (nitrocellulose) layer during the denaturing step. The advantage of collodion is that it can be removed by dipping the slide in ethanol-ether (1:1) before hybridization. If the collodion method proves reliable, it should permit more accurate quantitation, since the nuclei are in direct contact with the autoradiographic emulsion.

Discussion.—The results with *Xenopus* oocytes show that RNA can be hybridized with the DNA of cytological preparations under conditions that preserve the morphological integrity of the nucleus. The following features of the reaction indicate that we are dealing with true hybrid molecules. (1) The DNA must be treated with a denaturing agent to obtain the full reaction. (2) Prior removal of DNA by DNase eliminates the reaction. (3) The complex of RNA with the nucleus is stable to RNase. (4) The reaction is competed by unlabeled *Xenopus* RNA but is unaffected by heterologous *E. coli* RNA. (5) The reaction with rRNA is limited to the nucleoli of oogonia and to the amplified rDNA of oocytes. Its absence from normal diploid nuclei can be explained by the small amount of rDNA in these cells.

In the filter technique of Gillespie and Spiegelman,[5] contaminating basic proteins bind RNA nonspecifically. At the outset, therefore, we were concerned that nuclear histones would interfere with any cytological hybridization procedure. Our first experiments involved pronase digestion after formaldehyde fixation; however, we found such material difficult to denature, even though it retained good morphology. In the method described here, most of the basic proteins are removed by the ethanol-acetic fixative and the 45 per cent acetic acid treatment. This we have demonstrated by acrylamide gel electrophoresis, thus confirming the earlier experiments of Dick and Johns.[13]

Quantitation of our results has been difficult, since we have had no adequate control over self-absorption within the specimen and shielding by the agar layer. The effect of both factors was easy to demonstrate. Shielding was seen when the preparation was covered with 2 per cent agar instead of 0.5 per cent. In this case no autoradiograph was obtained. Self-absorption within the specimen was suggested by a comparison of the silver grains over the large diplotene nuclei and the more compact late pachytenes. Both contain the same amount of rDNA[12] and hence presumably hybridize the same extent; but the number of grains was greater over the larger, more flattened diplotene nuclei.

Keeping these complications in mind, we can make a rough estimate of the sensitivity of the technique. In autoradiographs exposed for one month, the

larger diplotene nuclei display approximately 3000 silver grains. These nuclei contain 25–30 pg of extrachromosomal DNA,[9, 12] of which about 18 per cent is complementary to 28*S* and 18*S* rRNA in filter experiments.[6] Thus, these preparations display 15 grains per day per picogram of hybridizable DNA. This can be translated into roughly 0.02–0.03 grain per day per nucleolus organizer.[6] At this level of sensitivity a single nucleolus organizer would be barely detectable after autoradiographic exposure for several months. For this reason the follicle nuclei in our preparations are negative (Fig. 1*C*).

The sensitivity of the hybridization technique can probably be increased by the use of RNA made *in vitro*. We are now preparing RNA enzymatically, using the *Xenopus* rDNA satellite as template. If we can increase the specific activity of the RNA by a factor of 10 or 100 over what is now available, we should be able to demonstrate the locus of rDNA in metaphase chromosomes of most higher organisms.

It should be relatively easy to demonstrate the rDNA in the polytene chromosomes of Diptera, which contain several hundred times as much DNA as a metaphase chromosome. Calculations are somewhat less reliable when dealing with other types of RNA. The genes for transfer RNA and for 5*S* ribosomal RNA can possibly be localized under the favorable conditions afforded by polytene chromosomes. Certain special problems, such as the cytological localization of the mouse satellite DNA[14, 15] and the integrated form of the SV40 viral DNA[16] should be approachable. In the latter case, highly radioactive complementary RNA has already been used effectively in filter hybridization experiments. It is difficult to predict what success may be had with messenger RNA species; hopefully it may be possible to characterize mixtures of messengers from different tissues or from different developmental stages. For such experiments the polytene chromosomes of Diptera offer the best chance of precise cytological localization.

The technical assistance of Mrs. Cherry Barney is gratefully acknowledged.

Abbreviations: rRNA, ribosomal RNA; rDNA, the DNA sequences coding for rRNA; SSC, 0.15 *M* NaCl, 0.015 *M* Na citrate, pH 7.0; toluene-PPO-POPOP, 4 gm of 2,5 diphenyloxazole and 50 mg of 1,4 bis [2-(4-methyl-5-phenyloxazolyl)]-benzene in 1 liter of toluene.

* Aided by USPHS grants GM 12427 and GM 397 from the National Institute of General Medical Sciences.

[1] Hall, B. D., and S. Spiegelman, these PROCEEDINGS, **47**, 137 (1961).
[2] Nygaard, A. P., and B. D. Hall, *J. Mol. Biol.*, **9**, 125 (1964).
[3] Bautz, E. K. F., and B. D. Hall, these PROCEEDINGS, **48**, 400 (1962).
[4] Bolton, E. T., and B. J. McCarthy, these PROCEEDINGS, **48**, 1390 (1962).
[5] Gillespie, D., and S. Spiegelman, *J. Mol. Biol.*, **12**, 829 (1965).
[6] Gall, J. G., *Genetics*, in press.
[7] Conger, A. D., and L. M. Fairchild, *Stain Technol.*, **28**, 281 (1953).
[8] The culture was kindly furnished by Dr. Keen Rafferty, The Johns Hopkins University School of Medicine, Baltimore.
[9] Brown, D. D., and I. B. Dawid, *Science*, **160**, 272 (1968).
[10] Evans, D., and M. Birnstiel, *Biochim. Biophys. Acta*, **166**, 274 (1968).
[11] Gall, J. G., these PROCEEDINGS, **60**, 553 (1968).
[12] Macgregor, H. C., *J. Cell Sci.*, **3**, 437 (1968).
[13] Dick, C., and E. W. Johns, *Biochem. J.*, **105**, 46 P (1967).
[14] Kit, S., *J. Mol. Biol.*, **3**, 711 (1961).
[15] Flamm, W. G., M. McCallum, and P. M. B. Walker, these PROCEEDINGS, **57**, 1729 (1967).
[16] Sambrook, J., H. Westphal, P. R. Srinivasan, and R. Dulbecco, these PROCEEDINGS, **60**, 1288 (1968).